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CYTOTOXIC EFFECTS OF THE HERBICIDE 3-AMINO-1,2,4-TRIAZOLE ON *DAPHNIA PULEX* (CRUSTACEA: CLADOCERA)

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Chemical control of nuisance plants is usually the most economical method available (Meyer, 1966). This has made the use of herbicides a popular technique for control of plants in aquatic environments. However, the introduction of herbicides into aquatic systems has been shown to have an adverse effect on non-target organisms such as aquatic invertebrates. Thus Walker (1964, 1965) and Cowell (1965) have reported drastic reductions in pond invertebrate population levels following application of herbicides.

One of the more commonly used herbicides is Amitrole (3-amino-1,2,4-triazole). It was first introduced as a herbicide in 1954 under the commercial name of Weedazole by Amchem Products, Incorporated. Commonly referred to as aminotriazole, the compound is a white crystalloid of 84.1 molecular weight and 150–153° C melting point. This heterocyclic nitrogen compound with its highly stable S-triazole nucleus is quite soluble in water (Mason, 1969).

Several authors have undertaken herbicide bioassay research on aquatic invertebrates (for review see Bunting and Robertson, 1975). In the past these papers have dealt primarily with the acute effects which these chemicals have on selected species of the cladoceran *Daphnia*. Crosby and Tucker (1966), using first instar organisms, investigated the toxicity of 16 herbicides on *Daphnia magna* Straus. They found in 26-hour acute static tests at 21° C the median immobilization concentration (IC50) of amitrole to be 23 ppm. This compares favorably with the work of Findley (1969), who in using aminotriazole, an amitrole formulation with 50% active ingredient, found that 0-24 hr old *D. magna* had an IC50 of 53.5 ppm in 24 hour acute static tests at 20° C. She also found a drastic reduction in IC50 value, to 3.55 ppm, when the test period was extended to 48 hr (Findley, 1969). Chronic bioassay studies have shown that aminotriazole in concentrations as low as 3.5 ppm retarded growth and decreased fecundity. Findley (1969) found that over a ten day period the carapace length was significantly shorter in animals reared in 3.5 ppm aminotriazole. She further noted that 80% of the adults reared in 3.5 ppm aminotriazole solution failed to produce eggs (Findley, 1969). Bunting and Robertson (1975), extending the work of Findley, found *Daphnia pulex* Leydig to be more sensitive to aminotriazole than *D. magna*. They determined that 0–12 hr old *D. pulex* exposed under static conditions for 48 hr at 20° C reached median survival concentration at 15.5 ppm. They further note that *D. pulex* is more sensitive to reproductive damage than is *D. magna*. Temperature affects the rate at which the herbicide acts at 15 and 20° C. Thus, to obtain similar rates of survival it was necessary to double the aminotriazole concentration at 15° C (Bunting and Robertson, 1975).

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This study was undertaken to examine the cytotoxic effects of the herbicide 3-amino-1,2,4-triazole in concentrations of 10 ppm (0.1 mg/ml) on the common water-flea *Daphnia pulex,* and to examine possible modes of entry into the animal.

Materials and Methods

The original stock culture of *Daphnia pulex* Leydig, secured from Turtox National Biological Supply, was identified using the key and description of Brooks (1957). A single adult from this population was randomly selected and cloned to provide all the animals (parthenogenetic females) used throughout the course of these studies.

The *Daphnia* was fed following the modified procedures of Dewey and Parker (1964) and Findley (1969) using a suspension of *Scenedesmus obliquus* (Indiana University Culture Collection, Starr, 1964), along with Fleishmann's dry yeast. The water was filtered once through No. 1 Whatman filter paper and stored to be used as needed. Chemical analyses were performed each time the water was filtered using a Hach DR-EL (Hach Chemical Co., Ames, Iowa) direct-reading portable kit (Table I). The pH was measured using a Corning Model 7 meter and was found to be between 7.0–8.2, well within the normal pH range of 5.8 and 9.2 for *Daphnia* (Lowndes, 1952).

The specimens of *S. obliquus* were grown in 1.0 liter Erlenmeyer flasks containing 500 ml of sterile filtered pond water to which had been added 10 ml of Alga-Gro concentrate (Carolina Biological Supply Co). The algal cultures were maintained at 24 ± 1° C in a 16 hr photoperiod of Grow-lux (Fisher Scientific, Atlantic, Ga.) fluorescent lighting. The algae were allowed to grow until the cultures were a dark green in color giving a Spectronic 20 reading of greater than 0.60 absorbance at 680 mμ prior to use.

Young daphnids were reared and collected by placing 5–8 adult *Daphnia* in each of several 100 ml beakers containing 75 ml of filtered pond water with 5 ml of suspended *Scenedesmus* culture added as food. The beakers were placed in an environmental chamber at 24 ± 1° C with a 16 hr Grow-lux flourescent lighting photoperiod imposed. At given time intervals, adults were removed by pipette and placed in freshly prepared beakers. All glassware used was chemically cleaned (Findley, 1969).

All the tests conducted were static in design. A stock solution of herbicide was prepared by dissolving 0.5 gram technical grade (100% active ingredient) 3-amino-S-triazole (Amchem Products, Inc., Ambler, Pennsylvania) in 500 ml of the filtered pond water. From the stock solution a lethal concentration of 10

Table I

Chemical analyses of pond water.

Analysis for	Mean (ppm)	Range (ppm)
Alkalinity (total)	84.2	70–100
Calcium hardness	70.0	50–80
Magnesium hardness	22.8	10–40
Total hardness	90.7	85–100
Iron (total)	0.08	0.06–0.10

ppm (0.1 mg/ml) was prepared with filtered pond water. The above concentration was selected based on the field use of amitrole as noted by Crosby and Tucker (1966). The final concentration solution was prepared fresh daily. The stock solution was discarded and fresh solution prepared every five days.

Individuals in the age range of 0–24 hr (post brood chamber release) were selected for study because Breukelman (1932) and Sanders and Cope (1966) found young animals to be more susceptible to herbicides than older individuals.

Ultrastructure studies

Acute lethal experiments for examination of cytotoxic effects were conducted in 8 dram vials. Each replication consisted of 24 crustacean (0–24 hr post brood pouch release) placed in individuals vials of 25 ml solution of 3-amino-1,2,4-triazole (10 ppm) to which had been added 1 ml of suspended *Scenedesmus* culture. Each vial was checked hourly after the initial 12 hr period and any immobile individuals removed and fixed for electron microscopy. An animal was considered to be immobile if, upon tactile stimulation with a glass pipette, it did not swim away. Immobilization rather than death of the daphnids was selected because of the difficulty in ascertaining when death occurs.

In addition, time sequence exposure was performed on 0–24 hr old individuals. Approximately 60 specimens of *Daphnia* were placed into 150 ml of a 3-amino-1,2,4-triazole solution (10 ppm) in a wide-mouth 250 ml Erlenmeyer flask to which had been added 10 ml of suspended *Scenedesmus* culture. At 6, 9, 12 and 15 hr after initiation of the experiment approximately ten animals were removed and fixed for examination with the electron microscope.

Specimens of *Daphnia* were fixed for electron microscopy by immersing them in 1% osmium tetroxide in 0.2 M phosphate buffer at pH 7.4. After fixation at room temperature for two hr, the animals were rinsed in phosphate buffer at room temperature for one hr. The daphnids were dehydrated in a cold graded ethanol series followed by several changes of propylene oxide prior to infiltration and flat embedding in Epon 812.

Sections were cut using a diamond knife and a Porter-Blum (MT-1) ultramicrotome and picked up on naked 300-mesh and Formvar coated 150-mesh copper grids. Staining was accomplished with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965). Electron photomicrographs were prepared using an RC EMU 3H or Zeiss EM 9S transmission electron microscope.

Acute static bioassay

In an attempt to more critically ascertain the relationship between the timing of herbicide toxicity and the molt cycle, 0–2 hr old daphnids were employed. Three types of experiments were conducted: first, effects of continuous exposure over a 24 hr period on 0–2 hr old daphnids; secondly, effect of shorter term exposure to different age groups during the first 24 hr growth; and thirdly, effects of temperature.

In all studies individuals were placed in 8 dram vials filled with 25 ml of 3 amino-1,2,4-triazole (10 ppm at 24° C) with 1 ml of *S. obliquus* suspension added as a food source. The organisms were examined at half hour intervals to establish

the time of onset of immobility. Controls without herbicide were run simultaneously and experimental samples were corrected by subtracting the number of immobilized control animals from the number of immobilized herbicide exposed animals in corresponding samples. The best line for the data was fitted by least squares method of linear regression and the regression coefficient tested by one-way analysis of variance to see if it differed significantly from zero.

Results

Cytotoxicity experiments

Several deviations from normal fine-structure were evident in daphnids exposed to 3-amino-1,2,4-triazole (10 ppm) until immobilization. The most consistent of these alterations in cell structure was seen in the mitochondria, especially those of the more metabolically active tissue such as the body musculature. The body musculature of *Daphnia* has been extensively studied with the light microscope (Binder, 1931). The striated muscle of *D. pulex* resembles that described in the copepod (Bougligand, 1962). In longitudinal section the normal fine structure shows the typical striations of skeletal muscle, with the "A" and "I" bands clearly distinguishable and with an "H" band and a "Z" line bisecting them, respectively (Figs. 1 and 3). The sarcomeres are short and of uniform length, 3 μm in the relaxed state, with the myofibrils varying in thickness and separated by varying amounts of sarcoplasmic reticulum (Figs. 1–4) and mitochondria. Individual fibers of the body muscularis vary markedly in their fine structure especially in regards to size of fibrils, amount of sarcoplasmic reticulum, number and size of mitochondria, and amount of glycogen granules. Some cells, "Fibrillenstruktur" fibers (Hoyle, 1967), have extensive sarcoplasmic reticulum (SR). The myofilaments (MF) are separated into bundles and the myofibrils (MFB) each completely enclosed by the sarcoplasmic reticulum (Figs. 3 and 4). Other cells are "Felderstrucktur" fibers (Hoyle, 1967) and have little sarcoplasmic reticulum (Figs. 1 and 2). Intermediate forms are also observed in *Daphnia.* Muscle of the appendages (Fig. 3) is characterized by an extensive sarcoplasmic reticulum (SR) to myofibril ratio. The muscle fibers are multinucleate with the nuclei being peripheral in location and circular in section. Also located peripherally are large numbers of mitochondria (M) and interspersed extrafibrillar glycogen stores (Figs. 1–4; GS). These muscle mitochondria are polymorphic in shape (Figs. 1–4). For the most part they are much larger than their counterparts found anywhere else in *Daphnia,* being up to 1.2 μm in length. Each mitochondrion possesses a large number of closely packed inner membranes, or cristae, and a moderately dense ground matrix all enclosed by a continuous other membrane.

Upon immobilization of *Daphnia* the muscle mitochrondria examined expressed various degrees of alteration from normal to lysing (Figs. 5–7). Not all muscle cells within a single organism were affected nor were all mitochondria within a single fiber affected equally (Fig. 7). However, for the most part mitochondria of all cells of a single muscle were altered to some degree structurally. The most common alteration observed was folding (arrow) of the outer mitochondrial

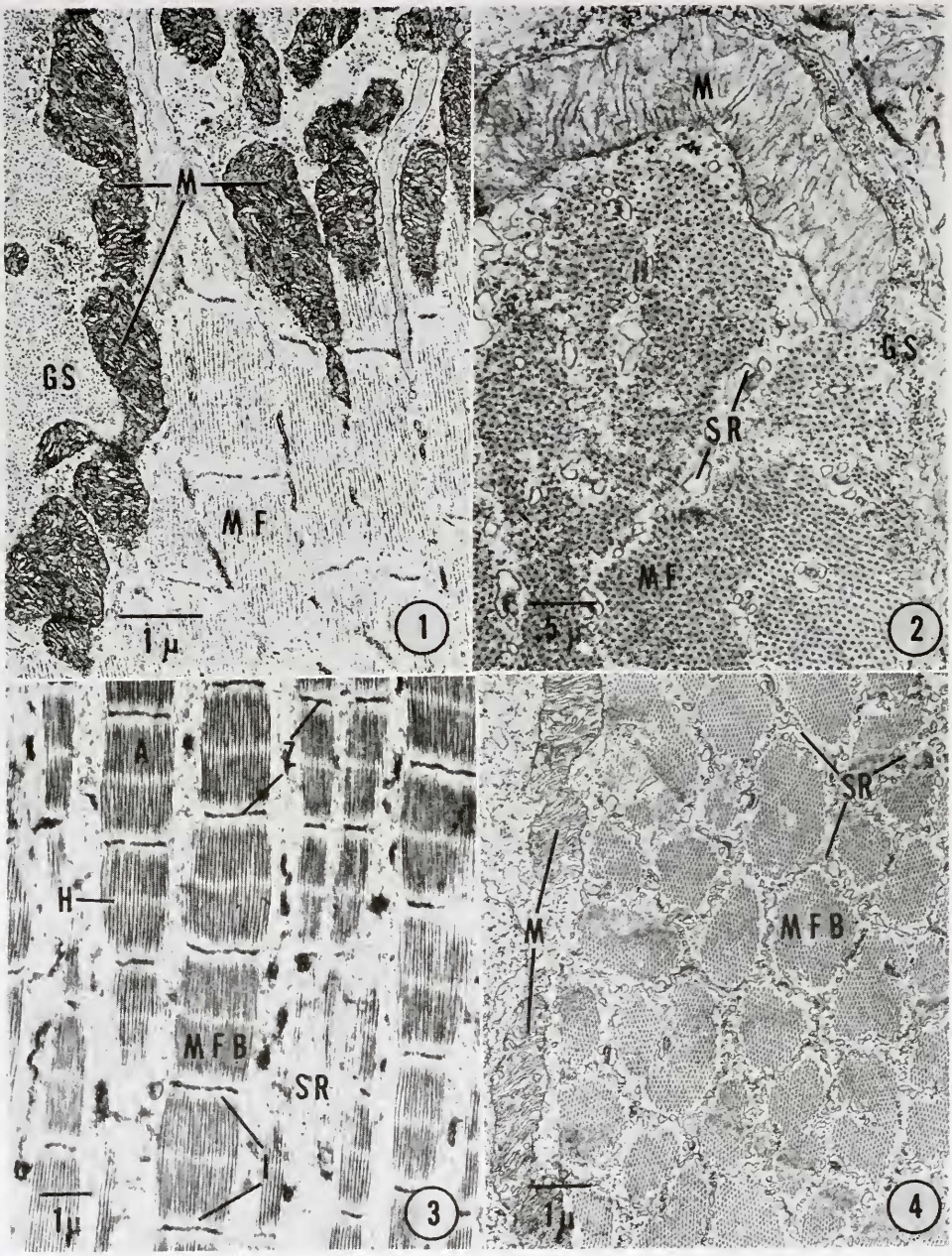

FIGURE 1. An oblique section through the edge of a normal "Felderstrucktur" muscle cell. Numerous large mitochondria (M) are located peripheral to the myofilaments (MF). Interspersed among the mitochondria are extensive glycogen stores (GY).

FIGURE 2. A transverse section through a normal "Felderstrucktur" muscle cell. Note the myofilaments (MF) are not seperated into descrete bundles by the sarcoplasmic reticulum (SR). A peripheral mitochondrion (M) and glycogen stores (GS) can also be observed.

membrane (Figs. 5–7). This was accompanied by organelle swelling and a reduction in electron density of the ground matrix and number of cristae (Fig. 5). Often observed was more extensive mitochondrial degradation characterized by gross shape alterations accompanied by drastic reduction in cristae number and matrix content (Fig. 6). Moreover, a few mitochondria were lysed (Fig. 11, LM). A small percentage of the muscularis mitochondria within a cell otherwise altered by 3-amino-1,2,4-triazole were observed to be only slightly affected, if at all (Fig. 7). Similar mitochondrial damage was observed in other cell types, though much less regularly. This was most often noted in the hypodermis (Fig. 8) and eye (Fig. 9) and was usually characterized by mitochondrial swelling. For comparison, inserts contain normal mitochondria from comparable regions of control animals.

In addition to mitochondrial alteration other cytotoxic effects were observed. These included disarrangement of the fibrils and myofilaments (MF) of the muscle cell, with the sarcoplasmic reticulum (SR) showing a concomitant swelling (Figs. 10, 11). The median compound eye often showed swelling and disassociation of the membranes enclosing the pigment granules (Fig. 9, PG). The least affected organs, at least structurally, were the midgut and ovaries. The latter showed only slight mitochondrial damage, while the former occasionally expressed alteration in cell shape and microvilli.

The cytotoxicity observed in *D. pulex* exposed to 3-amino-1,2,4-triazole (10 ppm) until becoming immobile is the same for 0–24 hr old and adult animals. While there was a wide range of structural alterations expressed by a variety of cell types, a general trend did appear. Mitochondrial damage was by far the most consistent structural change noted in the test animals and was often (50% of the time) the sole alteration observed. At no time during the course of the investigation were additional cytotoxic effects such as myofibril, sarcoplasmic reticulum or microvilli damage noted without accompanying mitochondrial swelling.

Mobile 0–24 hr old daphnids, exposed to the herbicide (10 ppm at 24° C) for up to 15 hr, showed no cell or organelle damage regardless of length of exposure. Reproductive-age specimens of *D. pulex* were also exposed to 3-amino-1,2,4-triazole (10 ppm, 24° C) until immobilization. Upon examination cytotoxic alterations comparable to those in 0–24 hr old animals were observed.

Acute static bioassay

During the course of the acute static tests and exposure for cytotoxicity studies (10 ppm 3-amino-1,2,4-triazole at 24° C) the 0–24 hr old and adult *D. pulex* expressed a change in behavior pattern. Prior to the onset of immobilization a decrease in the rate and efficiency of the antennal stroke or swimming move-

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FIGURE 3. A longitudinal section through a normal "Fibrillenstrucktur" muscle cell. The myofibrils (MFB) are parallel and generally of equal width. They are separated by extensive sarcoplasmic reticulum (SR). Note the typical banding pattern of an "A" band (A) and "I" band (I) bisected by a "H" band (H) and "Z" line (Z), respectively.

FIGURE 4. A transverse section through a normal "Fibrillenstrucktur" muscle cell. The numerous bundles or myofibrils (MFB) are separated from each other by the extensive sarcoplasmic reticulum (SR). At the edge of the cell two mitochondria (M) can also be observed.

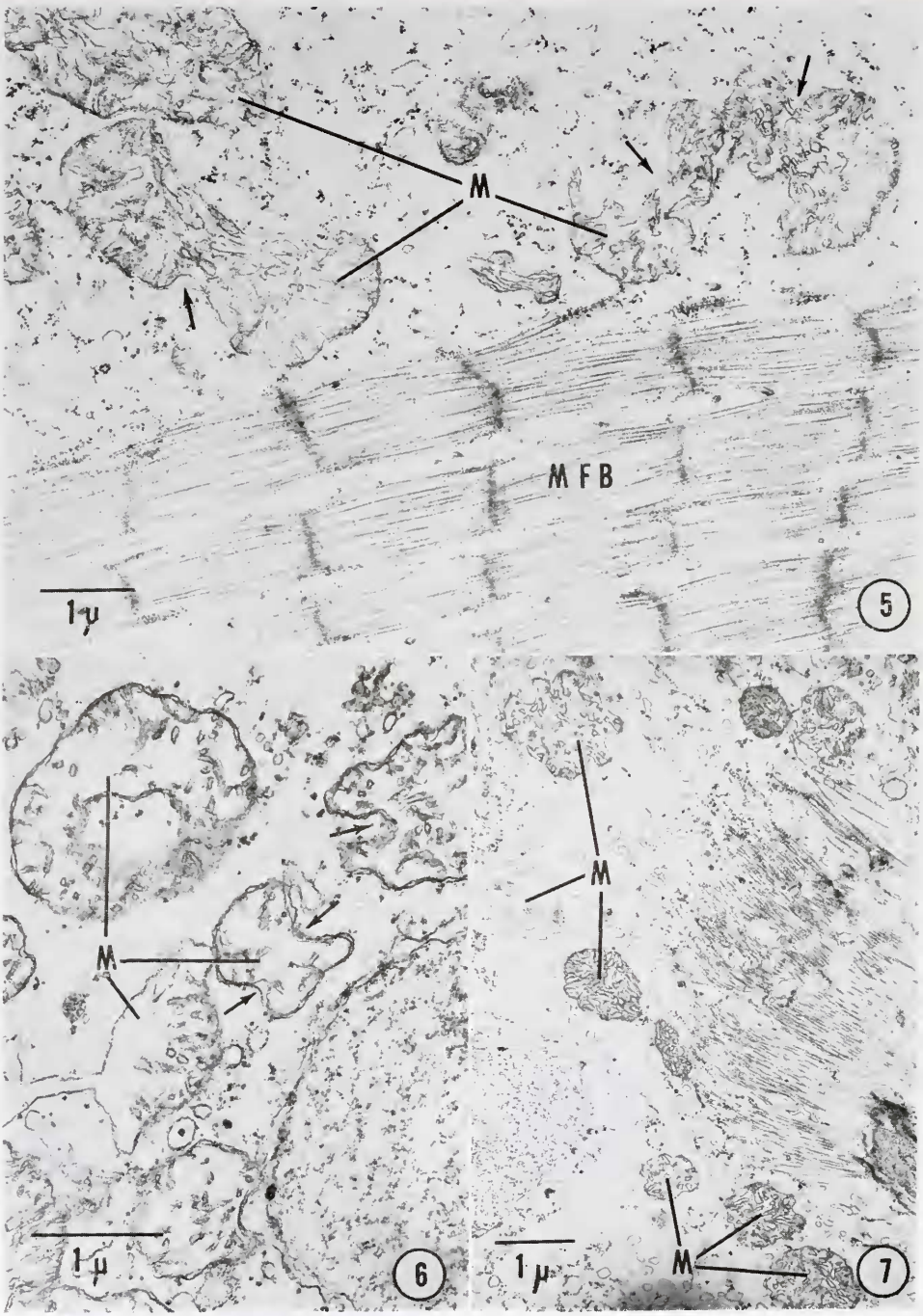
M
MFB
1μ
5
M
1μ
6
M
1μ
M
7

TABLE II

Daphnia pulex *immobilization numbers for 23-hour observations experiment of 0–2 hour animals exposed to 10 ppm 3-amino-1,2,4-triazole at 24°C. Total number of animals exposed = 81.*

Age ± 1 hour	Length of exposure	Total number immobile	Per cent immobile
18	17	0	0
19	18	12	14.8
19.5	18.5	15	18.5
20	19	28	34.6
20.5	19.5	45	55.5
21	20	61	75.3
21.5	20.5	69	85.2
22	21	78	96.3
22.5	21.5	81	100

ment was observed. The animals sank to the bottom of the vials and often rested on their sides. In 0–16 hr old animals this was consistently between 15 and 22 hr after continuous exposure. In the vast majority of cases when an individual was found to be immobile an exuvium was also seen in the vial.

To investigate the relationships between length of exposure and time of immobility specimens of *D. pulex* were exposed continuously to 3-amino-1,2,4-triazole. Animals 0–2 hr old were individually exposed (10 ppm at 24° C) and the time and number of immobile daphnids noted (Table II). No animals tested became immobile prior to 17 hr of exposure (18 ± 1 hr of age) and all individuals tested were immobile prior to 21.5 hr of exposure (22.5 ± hr of age). Thus none of the 81 animals tested became immobile prior to the projected mean first molt time for *D. pulex* at 24° C (16.73 hr. of age) based on the temperature development time curve (Bunting, 1973) for this species. A least squares linear regression of age (Y) versus % immobile (X) gives the equation $Y = 18.41 + (0.038)X$, and calculated mean immobilization time (IT 50) of 20.32 hr with a 0.86 hr standard deviation. None of the control animals became immobile. These data suggest that the time of immobility is closely related to molt. It also suggests a delay in time of ecdysis.

Since no immobility occurred until 18 hr of age in animals exposed to herbicide continuously after release from the brood pouch (Table II), the effect of exposure at different ages was examined. Zero to two hr old specimens of *Daphnia* were collected. Some were exposed immediately to a 3 amino-1,2,4-triazole (10 ppm at 24° C) while others were allowed to grow for intervals of 4, 8, 12, 16, 18, 20 or 24 hr before being placed in the test solution. All animals were monitored at one

FIGURE 5. A longitudinal section through a contracted muscle cell of an animal immobilized by 3-amino-1,2,4,-triazole. The mitochondria (M) have fewer cristae than in mitochondria of control daphnids and the matrix is less electron dense. The outer membrane of the mitochondrion is often folded (arrows). The myofibrils (MFB) are normal in appearance.

FIGURE 6. A section through the peripheral region of a muscle cell of a *Daphnia* immobilized by 3-amino-1,2,4,-triazole. More extensive damage to the mitochondria (M) was often observed. The number of cristae and electron density of the matrix are drastically reduced with folding (arrows) of the outer membrane.

FIGURE 7. A section through a muscle cell of a daphnid immobilized by 3-amino-1,2,4,-triazole. Note the variation in damage to the mitochondria (M) within a single cell.

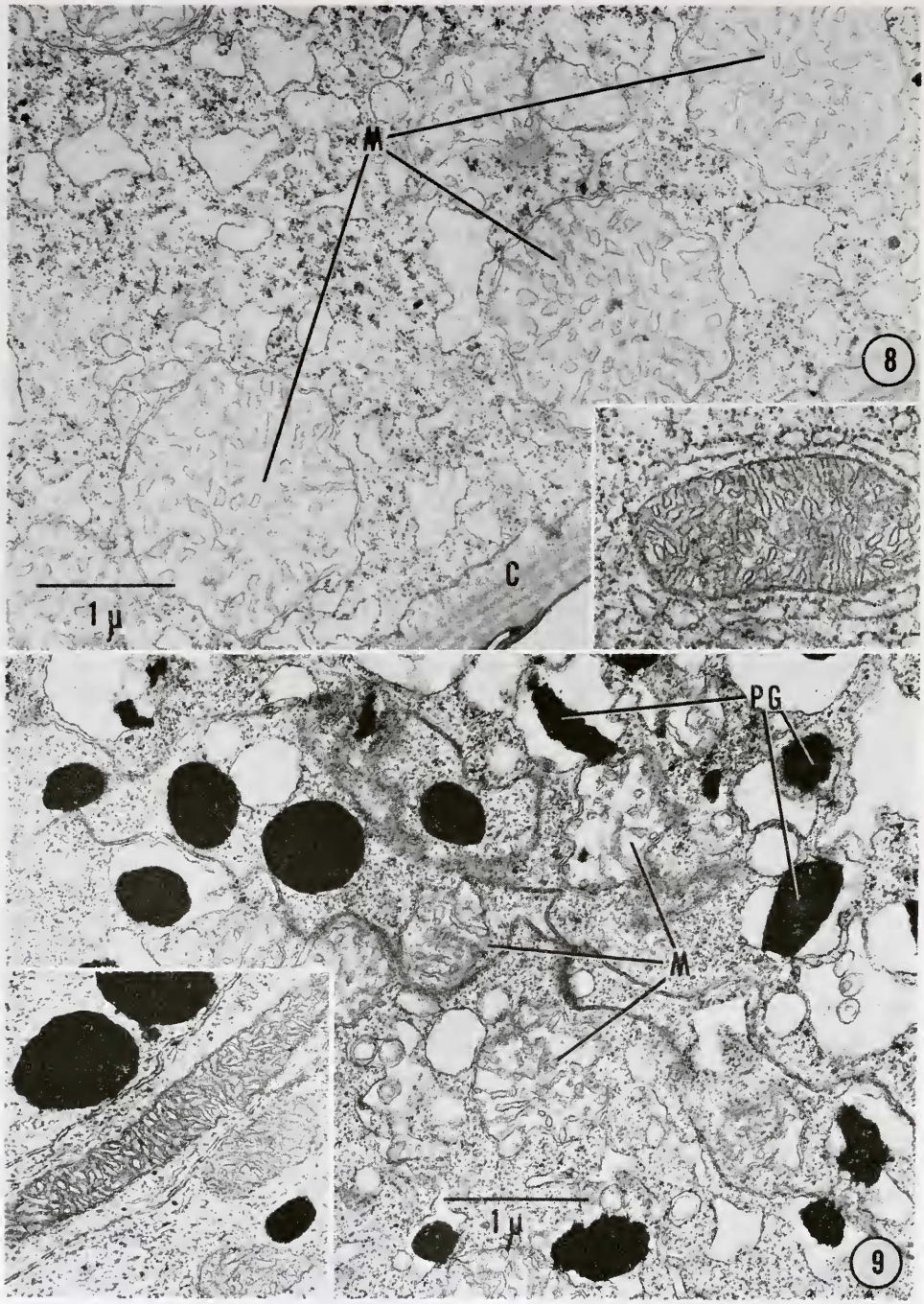

FIGURE 8. A section through the integument of an organism immobilized by 3-amino-1,2,4-triazole. The chitinous intima (c) is not altered. The mitochondria (M) of the cellular

hr intervals until immobile. Parallel controls were run and all experimental samples corrected as indicated previously. The length of time required for 95% immobility of the test animals (Y) versus the age of the animal at the time of exposure (X) was plotted (Fig. 12).

The length of exposure required to obtain immobility is inversely related to age up to 16 hr post-brood pouch release. Animals 0–2 hr old (1 ± 1 hr) required 21 hr exposure to herbicide to produce 95% immobility while animals 16 hr old (± 1 hr) when placed in the herbicide became immobile after only 6 hr exposure. Thus, as daphnids approached molt, the length of exposure required to immobilize them was reduced indicating an increased sensitivity to the herbicide. Animals placed in herbicide at 18 hr of age show a reduced sensitivity as indicated by the increase in exposure time over 16 hr animals for the same degree of immobility to be produced.

If 18 hr old specimens of *Daphnia* are post-molt animals as suggested by the data of Bunting (1973), then it would appear that sensitivity to the herbicide is directly related to the events of premolt. This is further supported by the effects of herbicide on 20 and 24 hr old animals. These two groups show a reduction in time required for 95% immobilization, which is equivalent to the time they have progressed in their growth period toward the second molt.

The effect of temperature on mobility of 0–24 hr old *D. pulex* exposed to 3-amino-1,2,4-triazole was examined. Animals were exposed to the herbicide (10 ppm) for a 24 hour period at 12, 16 and 24° C. At the end of this period, the percentage immobility was calculated for each temperature. A least squares linear regression of corrected percentage immobility (Y) versus log temperature centigrade (X) was plotted (Fig. 13). The regression coefficient of the calculated line $Y = 254 + (242)X$ was found by a one-way analysis of variance to be significantly different from zero at the 0.05 level. Animals subjected to the lower temperatures were less affected by the 3-amino-1,2,4-triazole as indicated by a mean percentage immobile of 6.35 and 39.80 for 12 and 16° C, respectively, as opposed to a mean immobility for 24° C of 79.65 %. Those replications expressing zero percentage immobile after the initial 24 hour exposure at 12° C were monitored for a second day with identical results of zero percentage immobility being observed. Thus, temperature affects the percentage mobility of 0–24 hour *Daphnia* exposed to 10 ppm 3-amino-1,2,4-triazole for 24 hr.

Discussion

Immobility of juvenile (0–24 hr) *D. pulex* exposed to 3-amino-1,2,4-triazole appears to be tied to the molt cycle. As test animals approach ecdysis, which based on the temperature-development time curve of Bunting (1973) is 16.73 hr

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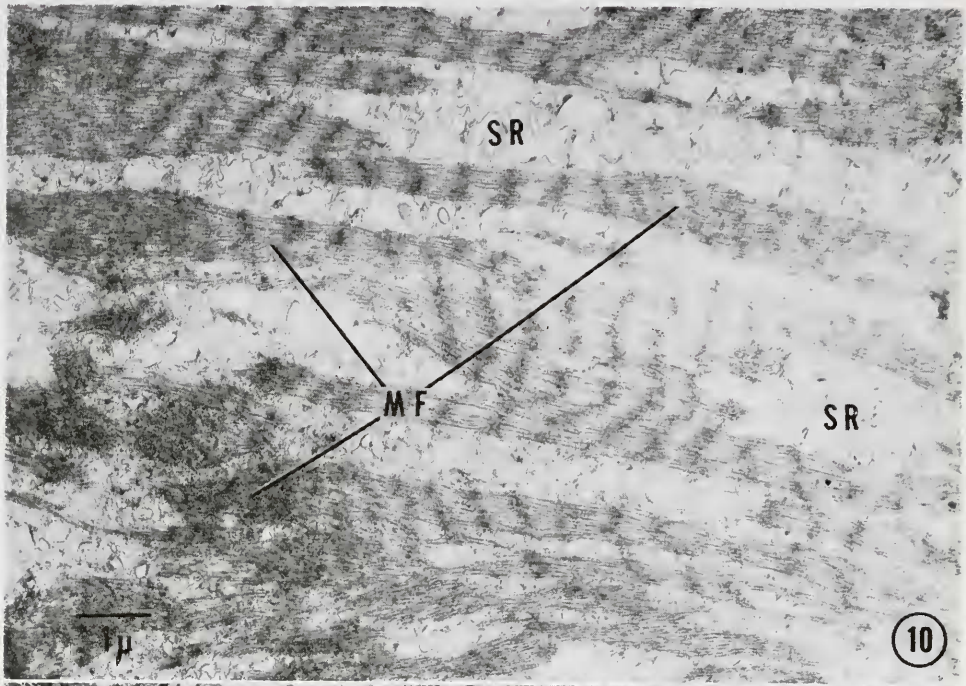
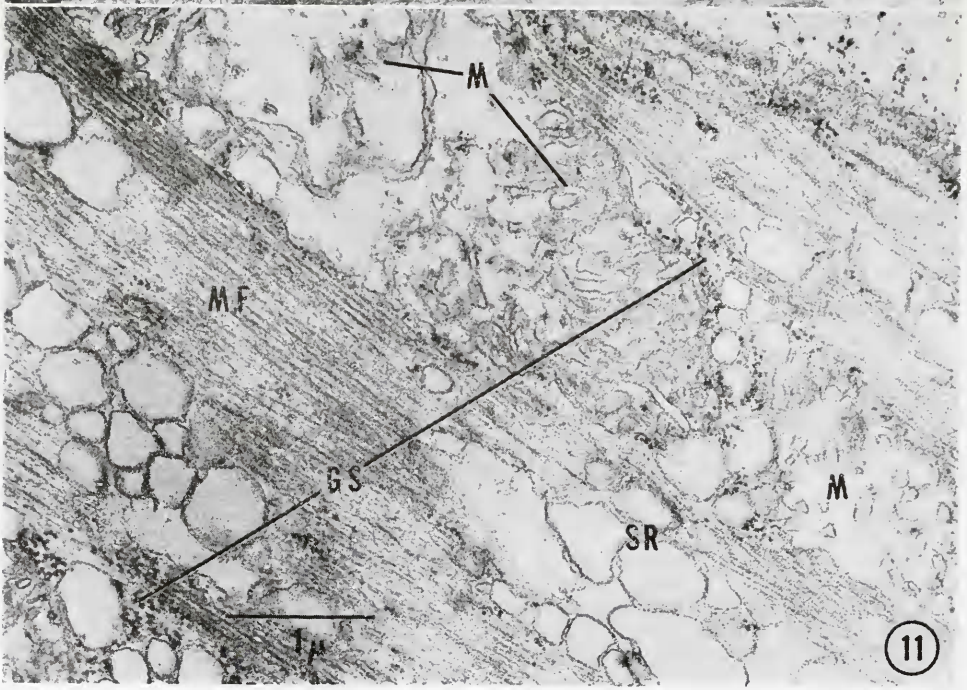
hypodermis are swollen. This is accompanied by a reduction in electron density of the matrix and number of cristae. The insert shows a mitochondrion of a normal hypodermal cell at a comparable magnification.

Figure 9. A section through a receptor cell from the median compound eye of an animal immobilized by 3-amino-1,2,4,-triazole. The mitochondria (M) have fewer cristae with less electron dense matrix and show a concomitant swelling. The pigment granules (PG) are separated from their encapsulating membrane. The insert shows pigment granules and mitochondrion of a normal receptor cell at a comparable magnification.


SR
MF
SR
1μ
10


M
MF
GS
M
SR
1μ
11

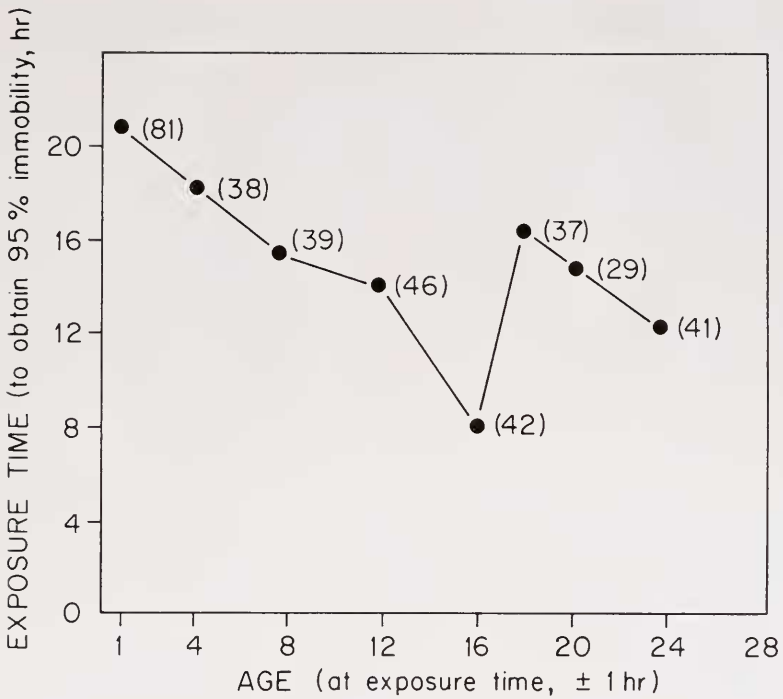


FIGURE 12. Effect of age at time of exposure to 10 ppm 3-amino-1,2,4,-triazole on the length of exposure required to obtain 95% immobility of 0-2 hr old *Daphnia pulex*. Number in parenthesis equals number of animals.

of age at 24° C, the length of exposure required for immobility decreases. However, once ecdysis is passed the required exposure time increases only to again decrease as the animals near their second molt. This is corroborated by the 6–15 hr sequential ultrastructure studies which showed no detectable organelle damage, including mitochondria, in first juvenile instar (0–24 hr old) daphnids exposed to the herbicide for up to 15 hr. Furthermore, the physiological state of intermolt adult *D. pulex* is not altered by exposure to 3-amino-1,2,4-triazole (10 ppm at 24° C) for 24 hr as indicated by the lack of alteration in the rate of oxygen consumption (Schultz, unpublished data).

Reduced temperature causes a reduced rate at which the herbicide acts on *Daphnia*. This is indicated by the fact that only 4 out of 51 test organisms were immobile after 24 hr exposure at 12° C, while at 16° C, 27 out of 70 animals tested became immobile. This is in agreement with the studies of Bunting and Robert-

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FIGURE 10. A longitudinal section through a muscle cell of an animal immobilized by 3-amino-1,2,4,-triazole. The myofilaments (MF) show varying degrees of disorientation as the banding pattern is lost. The sarcoplasmic reticulum (SR) is swollen.

FIGURE 11. An oblique section through a muscle cell of a daphnid immobilized by 3-amino-1,2,4,-triazole. The mitochondria (M) are damaged, some to the point of being lysed. The myofilament (MF) are disarranged and the sarcoplasmic reticulum (SR) is swollen. Note the interfibrilar glycogen stores (GS).

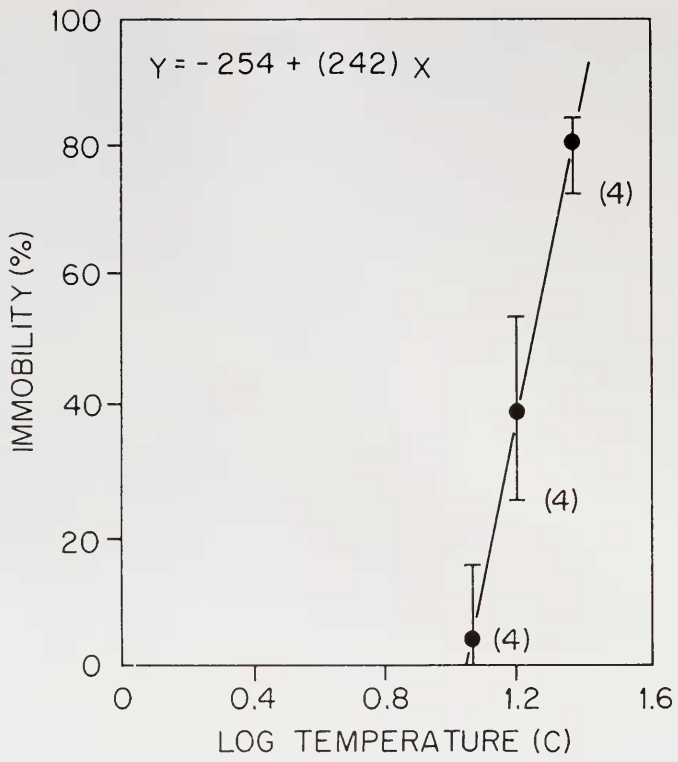

FIGURE 13. Effects of temperature on mobility of 0-24 hr old *Daphnia pulex* exposed to 10 ppm 3-amino-1,2,4-triazole. A least squares linear regression of corrected % immobility (Y) versus log temperature centrigrade (X). Number in parenthesis equals number of replications. Vertical bars indicate the range.

son (1975) who found that, in order to obtain similar rates of immobility at 15 and 20° C, it was necessary to double the amount of herbicide at the lower temperature. This may be correlated with a reduction in metabolic rate and longer instar duration at lowered temperatures, since Robertson (1971) reported that the mean first pre-adult instar duration of approximately 50 hr at 15° C was extended to 100 hr when the temperature was reduced to 10° C. Thus, at 12° C no animals would be expected to reach ecdysis (in fact even after 48 hr exposure), while at 16° C those animals which became immobile could represent the number reaching ecdysis. The time lag between projected time of ecdysis and time of immobility may be explained by a change in metabolic rate suggested by behavior changes which more closely approximate ecdysis.

The effect of 3-amino-1,2,4-triazole on plants has been extensively studied. It is readily absorbed through the foliage or roots and translocated throughout the plant (Ashton and Crafts, 1973). It causes chlorosis and inhibits regrowth from buds (Ashton and Crafts, 1973; for more in depth study see Hall, Johnson and Leinweber, 1954). Chlorosis is apparently due to the triazole interfering with light-induced changes in the proplastid resulting in a reduction in chlorophyll

synthesis (Mason, 1969). Mason (1969) further notes that amitrole affects protein and purine metabolism, flavin synthesis and acts as an enzyme inhibitor.

The most striking phyotoxic symptom of 3-amino-1,2,4-triazole exposure is albinism caused by chlorophyll destruction and impairment of chlorophyll synthesis. The herbicide also blocks light-induced plastid development in wheat seedlings (Bartels, 1965). Treated plastids lacked normal granafret membrane systems as well as chloroplast ribosomes (Bartels and Weier, 1969). All of these effects take a relatively long time to culminate and either do not apply to, or can not explain the sudden immobilization observed in *Daphnia*.

However, in addition to imidazoleglycerol phosphate dehydrase inhibition in protein metabolism, 3-amino-1,2,4-triazole has been found to block several other enzyme reactions (Hein, Appleman and Pyforna, 1956; Margoliash and Novogrodsky, 1958). This, plus catalase and fatty acid peroxidase irreversible inhibition (Castelfranco and Brown, 1963), has led to the suggestion that the herbicide undergoes a one-electron oxidation to the free radical which then attacks various enzymes, causing irreversible inhibition (Castelfranco and Brown, 1963). If such were the case, it may explain the effects expressed in *Daphnia* mitochondria, since these organelles appear to be rather sensitive sites for a variety of toxic agents. For example, Kennedy and Elliott (1970) observed that tobacco cigarette smoke caused disruption of mitochondria in *Tetrahymena pyriformis*. Gray and Kennedy (1974) reported that nontobacco cigarette smoke had a similar effect on *T. pyriformis* mitochondria. They extended their studies to show that cellular respiration was also effected. Other pollutants particularly $HgCl_2$ (Grityka and Trump, 1968; Tingle, Pavlat and Cameron, 1973) have also been reported to cause gross swelling of mitochondria, decreased density of the ground matrix and deterioration of cristae. Fox and Penner (1965) in studying the effect of 3-amino-1,2,4-triazole on inhibition of tricarboxylic acid cycle substrate oxidation by isolated cucumber mitochondria noted that concentrations of 10^{-5} to 10^{-3} reduced succinate utilization in a stepwise manner but failed to affect α-ketoglutarate utilization appreciably. Lotlikar, Remmert and Freed (1968), stated that amino-triazole in concentrations as high as 10^{-2} M had less than a 5% inhibition on oxygen uptake in cabbage mitochondria when tested for 90 minutes at 30° C. Thus, the similarities of function between mitochondria and chloroplasts and their general sensitivities to 3-amino-1,2,4-triazole is not surprising. The major variation seems primarily to be associated with their different rates of action.

The overall problem in *Daphnia* appears to be one of permeability. As long as the animal is in intermolt state the rate of entry of the herbicide from a 10 ppm solution may be slow enough that the 3-amino-1,2,4-triazole may be detoxified or eliminated without expressing any physiological or cytotoxic effects. However, during the stress of ecdysis with volume increase produced by uptake of water into the cells and the change in the permeability of the body surface (Lockwood, 1967), in the influx of 3-amino-1,2,4-triazole is probably increased. Since molting in *Daphnia* is considered to be a continuous process (Procella, Rixford and Slater, 1969), it is difficult if not impossible to pinpoint the time of permeability change associated with ecdysis. This does not exclude uptake of the herbicide by the gut *via* oral and anal drinking (Fox, 1952), but since the digestive tract shows minimal damage in experimental animals this mode of entry seems unlikely. In any

case, once in the animal, 3-amino-1,2,4-triazole probably inhibits enzyme reactions possibly including specific ones involved in oxidative phosphorylation thus stopping ATP synthesis and causing mitochondrial swelling. Such actions affect the most active tissue or enzyme system first, thus accounting for the consistent mitochondrial damage observed in muscle cells, the observed decreased rate and efficiency of antennal strokes and the sinking to the bottom of the vial expressed by treated animals just prior to immobilization. Disorientation of myofibrils, swelling of the sarcoplasmic reticulum, and other cell alterations are probably secondary effects caused by the breakdown of the osmoregulatory mechanism due to the lack of an external energy source, ATP. This theory is supported by the facts that the onset of immobility is: correlated with ecdysis; affected by age at time of exposure; and reduced with a reduction in temperature. It also explains the specificity towards mitochondrial damage in high energy requiring cells and the variation in other cell and tissue alteration observed in thin section.

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Summary

Zero to 24 hour old specimens of *Daphnia* were exposed to 10 ppm 3-amino-1,2,4-triazole at 24° C until immobilized. The most consistent alteration in cell structure was expressed by the mitochondria especially those of muscle fibers. Not all muscle cells within a single animal nor all mitochondria within a single cell were affected equally. The most common alteration observed was folding of the outer membrane. This was accompanied by organelle swelling and a reduction in electron density of the ground matrix and number of cristae.

In addition other cytotoxic effects were observed. These included general tissue swelling, disarrangement of myofilaments and dissociation of membranes. Mobile 0–24 hr old daphnids which were exposed to the herbicide for up to 15 hr showed no cell or organelle damage regardless of length of exposure.

Data from acute static experiments suggest that the time of immobility is closely related to molt for 0–24 hr juveniles.

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